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TITLE: Use of Synthetic Antibodies Targeted to the Jak/Stat Pathway in Breast Cancer

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14. ABSTRACT This proposal seeks to generate sABs (synthetic antigen binders) against the PRLr (prolactin receptor)signaling complex to systematically inhibit and modulate its important activities in breast cancer. This complex has clearly been demonstrated to play a significant role in development and spread of this disease, and yet the generation of pharmacologic agents that can specifically block their function has been slow. We hypothesize that sABs can be generated to the PRLr, CypA, and CypB proteins and delivered into the cell, where these reagents will block breast cancer growth, survival and spread. We have successfully generated sABs to CypA and B and the extracellular domain of PRLr and have begun their characterization. It is the intent of this proposal to validate the efficacy of these highly innovative reagents on a panel of breast cancer cells in vitro, with future translation into pre-clinical testing.					
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Table of Contents

	Page
Introduction	4
Body	4
Key Research Accomplishments	6
Reportable Outcomes	6
Conclusion	7
References	7
Appendix	8

Introduction and Scope of Research: Our research objective is to develop a novel set of technologies that will target the Jak/Stat signaling cascade in breast cancer (1). These technologies, which involve a new class of affinity reagents and intramolecular delivery tools, have the capability of identifying the most important nodes in this signaling pathway and ultimately inhibiting or modifying them to influence effects on breast cancer cell proliferation and death. The interplay between the Jak and Stat components in cytokine signaling has been an area of intense investigation. However, although many of the molecular interactions that occur between the m and with other signaling partners have been broadly implicated in breast cancer, they are poorly characterized because of a lack of appropriate experimental tools. Consequently, a host of basic questions remain to be answered. Our goal is to develop an experimental framework to sort out the most important interactions in the pathway and establish whether there is a specific Achilles Heel that can be exploited to attack breast cancers in innovative ways. As a long-term goal, we will utilize this information to develop novel synthetic antibody reagents that can be delivered with precision and potency to breast cancer cells (2).

Research Accomplishments: Aim 1- Generating synthetic antigen binders (sABs) to components of the prolactin receptor signaling network. We picked three components in the prolactin signaling pathway to focus on using sAB technology to: 1) block binding of prolactin (hPRL) to the extracellular domain (ECD) of its cognate receptor (hPRLr), 2) inhibiting CypA and 3) inhibiting CypB. CypA and CypB are proline isomerase enzymes that play critical roles in signaling; in the case of CypA to switch on the kinase activity of Jak2 and for CypB to assist in activation Stat5 in the nucleus (4, 5).

a) We have cloned, expressed and purified milligram quantities of CypA and CypB. We will employ phage display mutagenesis to generate sABs that inhibit the activity of these enzymes. We have developed a sorting protocol that selects sABs that block the active sites of these enzymes using competition with cyclosporinA (Figure 1). After round three, we picked 10 candidate sABs for each target (i.e. CypA and CypB) and evaluated their binding using surface plasmon resonance (SPR). sABs that bind to the enzymes with K_ds lower than 10 nM will be used in the biological assays described in Aim 2.

CypA & CypB

selective active site binding

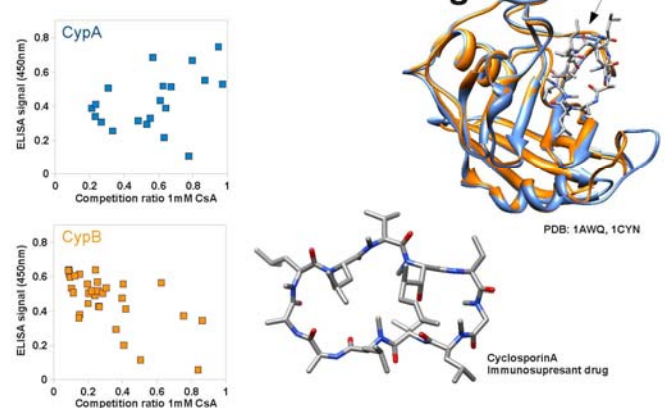


Figure 1- Phage ELISA data showing clones that interfere with cyclosporine binding indicating that they block the active site of the enzyme.

b) Using a novel phage display library (3), we have completed phage display selection for sABs that bind to the ECD of hPRLr at sites that interfere with hormone binding. We theorized that this class of sABs will inhibit receptor signaling in cell-based assays by antagonizing hormone binding. Four candidate sABs that met our criteria were evaluated for binding affinity using SPR. All the sABs had K_ds less than 30 nM, suggesting that they had potential application as potent hPRLr antagonists. To understand the mechanism through which the sABs might block hormone-receptor binding, we chose one of the sABs to determine a high resolution X-ray crystal structure analysis of the hormone-receptor complex. The structure is shown below.

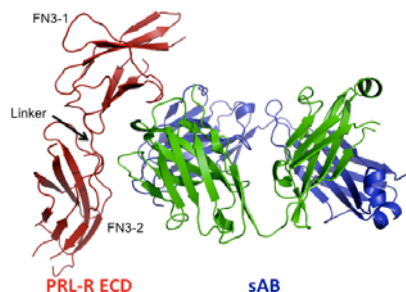


Figure 2- X-ray crystal structure of the hPRLR extracellular domain (ECD) bound to an inhibitory sAB. The receptor ECD has two fibronectin domains connected by a short linker. The sAB binds across the two domains. Interestingly, the known hormone binding site is actually on opposite face of the ECD. Thus, antagonism is generated through indirect effects, not direct blocking of the hormone binding site. The mechanism of hormone binding inhibition is based on the sAB altering the juxtaposition of the two fibronectin domains in a way that changes the hormone binding site. We note, that other inhibitory sABs might work through other mechanisms, like directly blocking the hormone binding site.

Aim 2- We will use these inhibitory sABs in cell-based assays to evaluate their effectiveness as receptor antagonists. Our hypothesis is that sABs that block hormone binding will have an inhibitory effect on downstream signaling as measured by Stat activation. To assess this, we have tested four inhibitory sABs in cell based assays to measure inhibition of Stat5 activation (Figure 3).

Cells were incubated with increasing concentrations of each sAB, or a control sAB against bacterial maltose binding protein. The effect of the sABs on prolactin signaling was determined using a dual luciferase luminescence assay under the control of a phospho-Stat promoter (Figure 3A).

The results indicate that 3 sABs (A8, A9 and A10) significantly inhibit prolactin signaling in a concentration dependant manner.

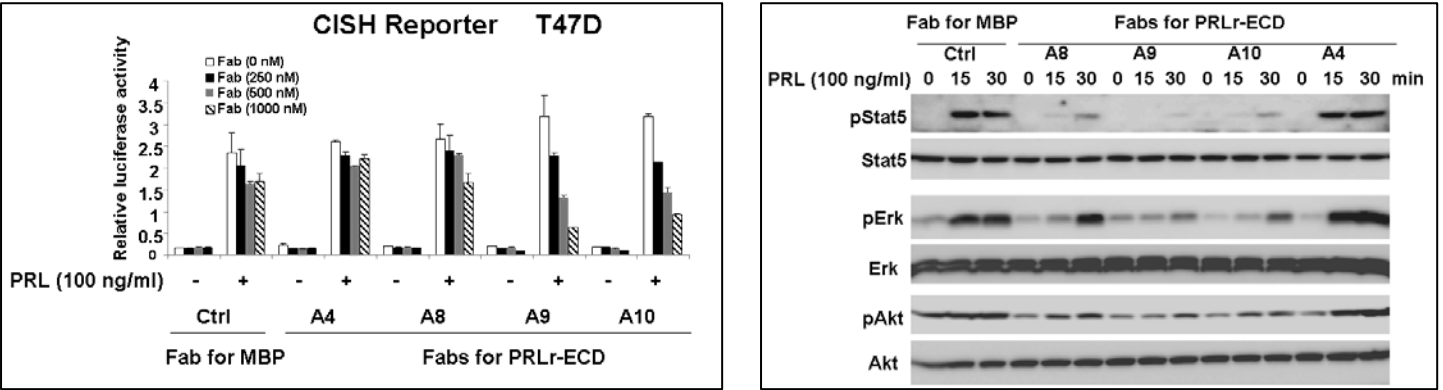


Figure 3- sAB inhibition of prolactin signaling. A) Effects on cell signaling based on luciferase expression. B) Measurement of decrease in phospho-Stat5 caused by the four individual inhibitory sABs.

To further examine the effect of the sABs on prolactin receptor signaling, we determined the change in the phosphorylation levels of various factors using western blot. While the control sAB (Fab for MBP) has no effect on prolactin signaling, sABs A8, A9 and A10 strongly inhibit phosphorylation of Stat5 and Erk (Figure 2B). Again, sAB A4 shows no effect on downstream signaling of prolactin as was observed in the luciferase assay.

sABs influence receptor internalization-

Many breast and ovarian tumors are characterized by the overexpression of the PRL-R. This triggers an overall enhancement of autocrine signaling through locally produced prolactin and down regulation of the signaling process by the internalization of the hormone-receptor complex. Thus, we examined the ability of the sABs to inhibit the internalization of prolactin and hGH through receptor endocytosis in T47D cells. This breast cancer cell line is known to express PRLR, which is rapidly internalized upon binding to hormone. T47D cells were incubated with 100 nM fluorescently-labeled

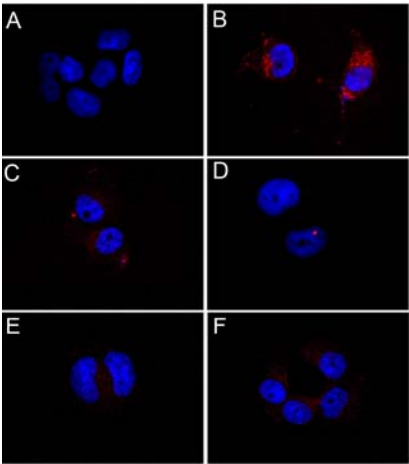


Fig. 4: Inhibition of internalization of prolactin by PRLR sABs. T47D cells were untreated (A) or treated with 100 nM prolactin-cy5 in the absence of sABs (B) or in the presence of 2 μ M sAB A4 (C), sAB A8 (D), sAB A9 (E) or sAB A10. All panels represent two merged channels; blue: DAPI nuclear stain, red: cy5.

prolactin or hGH in the presence or absence of 2 μ M sAB A4, A8, A9 or A10 for 4 hrs. Fluorescence microscopy was used to investigate the effect of the sABs on the ability of the cells to uptake the hormones. The images indicate that sABs A8, A9 and A10 significantly decreased or completely abolished the internalization

of prolactin, whereas sAB A4 had no effect (Fig. 4). sA B A4 also had no effect on the internalization of hGH (Fig. 5). In contrast with prolactin, hGH internalization was not completely inhibited by sABs A8, A9 and A10. This residual internalization of hGH is most likely carried out by the hGH receptor, which has been shown to be expressed in this cell line. Interestingly, sAB A9, which exhibits the highest affinity for the receptor had the greatest inhibitory effect on the internalization of both hormones. sAB A4, does not bind to the receptor and had little to no effect on the internalization of either hormone

Key Research Accomplishments

- Used phage display to select sABs that act as inhibitors to CypA and CypB.
- Generated and characterized a sAB against the PRLr-ECD and showed it was a potent inhibitor against PRL binding to the ECD.
- Determined the X-ray crystal structure of the sAB- PRLr-ECD crystal structure at 2.8Å resolution identifying the mechanism of inhibition.
- Established that sAB binding interferes with PRL binding through an allosteric mechanism.
- Demonstrated that Receptor-mediated delivery can effectively deliver functional sABs into the cytoplasm.

Reportable Outcomes

Publications-

Rizk SS, Luchniak A, Brawley CM, Rock RS, Kossiakoff AA. (2009) “An engineered substance P variant for receptor-mediated delivery of synthetic antibodies into tumor cells.” *Proc Natl Acad Sci USA*. 106:11011-5.

Duguid E, Zhang J, Rizk SS, Symborska A, Kouido J, Clevenger CV and Kossiakoff AA. “ The structure and function of a synthetic antibody- complexed to the extracellular domain of the prolactin receptor: signaling inhibition through an allosteric mechanism” (in preparation).

Meetings where aspects of the work were presented through invited talks

Protein Society Meeting, San Diego, Ca.

ESF-EMBO Research Conference, Madrid, Spain.

Baxter Drug Discovery Symposium (keynote speaker) San Francisco, Ca.

University of Chicago Biosciences retreat Galena, Il.

Gordon Research Conference on Biomolecular Interactions and Methods, Galveston, TX.

9th Annual Antibody Therapeutics Conference, San Diego, CA.

Cold Springs Harbor Conference- Protein Structure based drug design, Suzhou, China.

CHI Symposium on Molecular Medicine, San Francisco, CA.

Seminar Presentations

Georgia Tech- Distinguished Lecture Series in Systems Biology

Oxford University

Johns Hopkins University Department of Biophysics

University of Maryland, Baltimore Departments of Biochemistry and Virology

University of Kansas, Department of Bioinformatics and Computational Biology

University of Texas Southwest Department of Biochemistry
Northwestern University, Department of Biochemistry, Molecular Biology and Cell Biology
Purdue University, Department of Biology
Genentech, Inc., Department of Protein Engineering
California Institute of Technology, Department of Chemistry
University of Wisconsin, Department of Biochemistry.

Patents

Patent Pending: “Receptor-mediated delivery of bioactive cargos” AA Kossiakoff inventor. (see appendix)

Conclusions

The reagents that are being generated are of exceptional importance to both basic and translational science in that they will provide a means of delivering inhibitory probes intracellularly into viable cells and modulate their activity.

References:

- 1 -Clevenger,C.V. Role of Stat family transcription factors in human breast cancer, *Am J Pathol*, *165*: 1449-1460, 2004.
- 2-Rizk SS, Luchniak A, Brawley CM, Rock RS, Kossiakoff AA. An engineered substance P variant for receptor-mediated delivery of synthetic antibodies into tumor cells, *Proc Natl Acad Sci USA*. *106*:11011-5, 2009.
- 3- Fellouse,F.A., Esaki,K., Birtalan,S., Raptis,D., Cancasci,V.J., Koide,A., Jhurani,P., Vasser,M., Wiesmann,C., Kossiakoff,A.A., Koide,S. and Sidhu,S.S. High-throughput generation of synthetic antibodies from highly functional minimalist phage-displayed libraries, *J Mol Biol*, *373*: 924-940, 2007.
- 4- Rycyzyn,M.A., Reilly,S.C., O'Malley,K. and Clevenger,C.V. Role of cyclophilin B in PRL signal transduction and nuclear retrotranslocation, *Mol.Endocrinol.*, *14*: 1175-1186, 2000.
- 5- Rycyzyn,M.A. and Clevenger,C.V. The intranuclear prolactin/cyclophilin B complex as a transcriptional inducer, *Proc.Natl.Acad.Sci.USA*, *99*: 6790-6795, 2002.

1) Patent description for receptor-mediated delivery technology



The University of Chicago
Office of Technology and Intellectual Property

Receptor-mediated delivery of large (up to 400 kDa) bioactive agents to live cells; can deliver antibodies, peptides, DNA, RNA and nanoparticles

A fundamental challenge in developing effective therapeutic agents for a wide variety of diseases is achieving efficient, specific delivery of the agents to the affected tissues. While significant advances have been made in the development of peptide, protein and DNA or RNA therapeutics, targeted delivery of these bioactive agents remains a major obstacle in drug development.

Technology Description

Dr. Anthony Kossiakoff and colleagues have developed a unique and highly effective technology for delivering bioactive agents to the cytoplasm of live cells without compromising the integrity of the cell membrane. This receptor-mediated delivery technology is based on an 11 amino acid variant of substance P (SPv), a neuropeptide that has nanomolar affinity for neurokinin receptor-1 (NK1R). Bioactive cargos can be conjugated to SPv and are efficiently internalized by NK1R expressing cells. The internalized cargos are able to escape from the endosome, but still retain their biological activity. Dr. Kossiakoff and colleagues

have conjugated and successfully delivered a variety of different cargos to cells, including intact native and synthetic antibodies (sABs), other proteins and peptides, imaging agents, DNA, RNA (siRNA and shRNA), and nanoparticles (see Publication). Of particular note is that this technology can be used to deliver large protein payloads, up to 400 kDa, intact and functional to live cells.

Potential Benefit

NK1R is overexpressed in many types of tumors and primary cancers, including breast carcinomas, adenocarcinomas of the colon, astrocytomas, and glioblastomas. Consequently, this technology could have great therapeutic potential by allowing one to discriminate between cancer cells expressing the NK1R and normal cells. Further, the specific delivery of sABs directed toward cytoplasmic targets within tumor cells potentially changes the paradigm for antibody-based therapies. These therapies may no longer be limited to the current extracellular targets. Thus, using sABs designed to inhibit intracellular signaling nodes, the

possibility certainly exists for focusing future antibody therapies toward a much richer set of cancer targets.

Product: Pharmaceutical (delivery platform)

Development Stage: Optimize Lead / *in vitro* Testing

Primary Inventor: [Anthony Kossiakoff PhD](#), Ortho S.A. Sprague Professor and Chair, Biochemistry & Molecular Biophysics

Scientific Publication: [Rizk et al. PNAS. 2009 Jul 7; 106\(27\): 11011-5](#)

License Status: Available for licensing

Patent Status: 12/375,179 pending ([PCT WO 2009/076463 A1](#))

Reference: UCHI 1670

Contacts: Ben Dibling PhD, bdibling@tech.uchicago.edu, (773) 834-1270, or Thelma Tennant PhD, ttennant@tech.uchicago.edu, (773) 834-4020

2) Student posters at Biosciences Retreat Galena, IL 11/10- 11/11.

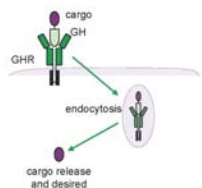
Specific delivery of biologically active cargo to cancer cells through growth hormone receptor

Anna Szymborska, Kossiakoff Lab

Specific delivery of drugs to cancer cells, without affecting healthy tissues, has been a bottleneck in cancer therapy. Receptor Mediated Delivery, a novel concept developed in Kossiakoff's Lab, takes advantage of the natural phenomenon of receptor endocytosis upon ligand stimulation in order to specifically deliver payload of therapeutic agents to cancer cells.

Growth hormone receptor (GHR) and prolactin receptor (PRLR), both induced by growth hormone (GH), have been reported to highly over express in cancer cells of several tissues, such as prostate, breast, ovary, liver. We are using growth hormone as a carrier for potentially lethal or therapeutic substances such as siRNA, miRNA, anti-sense DNA, plasmids, toxins, antibodies in order to deliver those substances to tumor cells.

We are also interested in producing antibody binding fragments (FAB) against prolactin and growth hormone receptors, that selectively block their binding sites and thus allow for studying of internalization process and signaling through one kind of receptor at a time. The FAB are selected from phage-displayed library.



Techniques:

- Protein engineering, expression in E. Coli and purification with diverse chromatography methods
- Phage display of antibody binding fragments
- Cell culture techniques
- FACS analysis, confocal microscopy, Western blotting
- Surface plasmon resonance

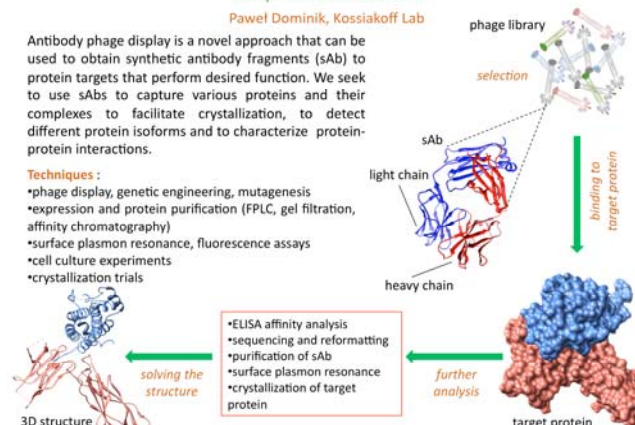
Antibody phage display as a novel approach for characterization of protein complexes and isoforms

Pawel Dominik, Kossiakoff Lab

Antibody phage display is a novel approach that can be used to obtain synthetic antibody fragments (sAb) to protein targets that perform desired function. We seek to use sAbs to capture various proteins and their complexes to facilitate crystallization, to detect different protein isoforms and to characterize protein-protein interactions.

Techniques:

- phage display, genetic engineering, mutagenesis
- expression and protein purification (FPLC, gel filtration, affinity chromatography)
- surface plasmon resonance, fluorescence assays
- cell culture experiments
- crystallization trials



SPECIFIC TRANSPORT OF BIOMOLECULAR CARGO USING RECEPTOR-MEDIATED DELIVERY

Agnieszka Misiura, Kossiakoff Lab

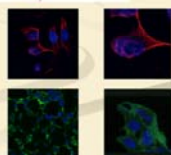
One of the biggest challenges in cancer treatment is to exclusively target the diseased cells, while leaving normal tissues unaffected. In the Kossiakoff's lab, we are developing Receptor-Mediated Delivery (RMD) as a novel method to efficiently and specifically transport therapeutic agents into cancer cells. RMD exploits highly specific ligand-receptor interactions and facilitates internalization of biomolecular cargo covalently attached to a ligand that targets a specific cell-surface receptor through endocytosis. This method takes advantage of the phenomenon that many diseased cells overexpress characteristic types of receptors as compared to normal tissues. I utilized substance P (SP), an 11-amino acid neuropeptide ligand of the NK1 receptor, as a delivery-vehicle of DNA encoding marker/therapeutic genes. SP binds with high affinity to the receptor and undergoes rapid internalization. Introduction of modifications to SP for covalent attachment of DNA does not interfere with the internalization process. Modified variant of substance P with nuclear localization signal was engineered for DNA targeting to the nucleus. NK1 receptor is highly overexpressed in many types of cancer, for example glioblastoma brain tumors, therefore SP has a great potential for use as a therapeutic agent.



Techniques:

- Confocal & Fluorescence Microscopy
- Flow Cytometry Analysis
- Solid-phase Peptide Synthesis
- Chemical Conjugation of Proteins
- Mass Spectrometry
- Western Blot
- Cell Culture
- Real Time PCR

Confocal microscopy was used to analyze the SP-DNA conjugate uptake (red) and GFP expression (green) by cells overexpressing NK1 receptor



Supporting Data- None